

A novel 65 kDa RNA-binding protein in squid presynaptic terminals

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Abbreviations: CID-MS/MS, collision-induced dissociation mass spectrometry; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetate; EPI-MS/MS, electrospray ion mass spectrometry; EST, expressed sequence tags; FPLC, fast protein liquid chromatography; hnRNP, heterogeneous nuclear ribonucleoprotein; MALDI-TOF/TOF-MS, matrix assisted laser desorption, time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate

Abstract A polyclonal antibody (C4), raised against the head domain of chicken myosin Va, reacted strongly towards a 65 kDa polypeptide (p65) on western blots of extracts from squid optic lobes but did not recognize the heavy chain of squid myosin V. This peptide was not recognized by other myosin Va antibodies, nor by an antibody specific for squid myosin V. In an attempt to identify it, p65 was purified from optic lobes of *Loligo plei* by cationic exchange and reverse phase chromatography. Several peptide sequences were obtained by mass spectroscopy from p65 cut from SDS-PAGE gels. BLAST analysis and partial matching with ESTs from a *Loligo pealei* data bank indicated that p65 contains consensus signatures for the hnRNP A/B family of RNA-binding proteins. Centrifugation of post mitochondrial extracts from optic lobes on sucrose gradients after treatment with RNase gave biochemical evidence that p65 associates with cytoplasmic RNP complexes in an RNA-dependent manner. Immunohistochemistry and immunofluorescence studies using the C4 antibody showed partial co-labeling with an antibody against squid synaptotagmin in bands within the outer plexiform layer of the optic lobes and at the presynaptic zone of the stellate ganglion. Also, punctate labeling by the C4 antibody was observed within isolated optic lobe synaptosomes. The data indicate that p65 is a novel RNA-binding protein located to the presynaptic terminal within squid neurons and may have a role in synaptic localization of RNA and its translation or processing.

Key words: myosin V; hnRNP complex; RNP motifs; synaptosome; optic lobe; *Loligo*

Regulated RNA translation systems are found in axons and presynaptic terminals as well as in dendrites and postsynaptic terminals of neurons where they control protein synthesis in response to specific needs of these subcellular domains (Giuditta et al., 2002; Schuman et al., 2006; Steward and Schuman, 2003; Pfeiffer and Huber, 2006; Sotelo-Silveira et al., 2006; Twiss and Fainzilber, 2009). Experiments on the squid giant synapses in the stellate ganglion and the large synaptosomes prepared from the photoreceptor neurons of squid optic lobes have demonstrated that the presynaptic terminal is endowed with an active system of protein synthesis (Crispino et al.,

1997;Crispino et al., 1993;Gioio et al., 2004;Jimenez et al., 2002;Kaplan et al., 2004). There is evidence to support the idea that a subset of neuronal mRNAs are selectively transported to and translated within the axon and the presynaptic terminal of these neurons (Crispino et al., 1997;Gioio et al., 2004;Kaplan et al., 2004). However, protein components and functions for regulated protein synthesis at the presynaptic terminal are not well characterized and need to be explored. Myosin V, an actin-based molecular motor ubiquitously found in eukaryotes, has been isolated and partially characterized from squid optic lobes and giant axons (Molyneaux et al., 2000;Cohen, 2001;Brown et al., 2004;Tabb et al., 1998). Myosin Vs represent one of the most well characterized classes of unconventional myosins (Langford, 2002;Trybus, 2008;Desnos et al., 2007). In vertebrate neuronal tissues, the paralog myosin Va (MVa) is localized at both pre- and postsynaptic sites and has been implicated in synaptic function and plasticity (Mani et al., 1994;Prekeris and Terrian, 1997;Walikonis et al., 2000;Takamori et al., 2006;Yoshimura et al., 2006;Correia et al., 2008). MVa and its mRNA have been identified in periaxoplasmic ribosomal plaques in axons (Calliari et al., 2002;Sotelo-Silveira et al., 2004) and, perhaps most remarkably, members of the myosin V class have been implicated in the transport and cytoplasmic localization of mRNA (Jansen et al., 1996;Yoshimura et al., 2006;Chang et al., 2008;Salerno et al., 2008). The exact function of MVa at the synapse has yet to be clarified. For this reason we investigated the possibility of using MVa antibodies as probes into the squid giant synapse, which is a model system for synaptic events. Serendipitously, we identified a novel 65 k, RNA- binding protein in the presynaptic terminals of squid nervous system.

Experimental procedures

Tissue preparation from squid

The optic lobes and stellate ganglia were dissected from freshly killed *Loligo pealei* obtained from the Marine Biological Laboratory in Woods Hole or from *Loligo plei* obtained from the *Centro de Biologia Marinha-CEBIMar*, University of São Paulo, São Sebastião, Brazil.

Synaptosomes were prepared according to Crispino (Crispino et al., 1997) from four optic lobes quickly dissected from decapitated squids and homogenized in 3 ml of ice-cold homogenizing medium, HM (0.7M sucrose in 20 mM Tris-HCl, pH 7.4) in a loose-fitting, motor-driven, Teflon-glass homogenizer by 8 slow up and down strokes. The homogenate was spun at 3,000 x g for 11 min at 4°C and the supernatant spun at 15,000 x g for 30 min. The floating layer containing the synaptosomes was collected, resuspended in HM buffer and spun at 15,000 x g for 20 min. The floating layer was again collected and resuspended in artificial seawater. For immunofluorescence studies both freshly prepared synaptosomes and dissected stellate ganglia were fixed in PLP buffer (phosphate buffered saline, pH 7.4, containing 10 mM sodium periodate, 75 mM lysine and 0.5% paraformaldehyde for 6-8 hrs at 4°C. Samples were subsequently washed 3 times for 10 min in phosphate buffered saline (PBS) at 4°C and treated as described below.

For purification and biochemical studies freshly dissected optic lobes were frozen in liquid nitrogen and stored at -70°C until used.

Antibodies

Polyclonal antibodies to the head domain (C4 and C5) and medial tail domain (α -MVaMT) of chicken myosin Va were raised in rabbits by inoculation with recombinant proteins, corresponding to aa 5-752 and aa 1117-1435, respectively (Espreafico et al., 1992; Costa et al., 1999). These antibodies were affinity purified on tissue-purified myosin Va (Cheney, 1998), immobilized on PVDF membranes. A polyclonal antibody against the C2A domain of squid synaptotagmin conjugated with rhodamine (anti-syt-C2A) was used to label synaptic vesicles (Fukuda et al., 1995; Mikoshiba et al., 1995). Secondary antibodies conjugated to alkaline phosphatase or peroxidase were obtained from Pierce (Rockford, IL) and to Alexa 488 from Molecular Probes (Invitrogen, Carlsbad, CA).

Purification by cationic and reverse phase chromatography

Approximately 4 g (40 lobes) of frozen optic lobes were homogenized in 60 ml of an 8 M urea solution in buffer A (40 mM Hepes, pH 7.6, 10 mM EDTA, 3 mM DTT and protease inhibitors 1 mM benzamidine, 2 µg/ml aprotinin and 0.3 mM PMSF) and centrifuged at 40,000 x g for 40 min at 4°C. The supernatant (S1) was diluted 1:1 volume in buffer A, without urea and centrifuged at 20,000 x g for 15 min at 4°C. The supernatant (S2) was applied to a 15 ml SP-Sepharose column (GE Health Science, Chalfont St. Giles, UK) equilibrated in buffer A and eluted by application of a linear gradient from 0 to 1.2 M NaCl in buffer A. Fractions containing p65 identified by western blot were eluted at 750-900 mM NaCl and pooled. The pooled sample was fractionated by FPLC (ÄKTA purifier, GE Health Science) on a Source 5RPC SR 4.6/150 column (GE Health Science) using a non-linear gradient of 0 to 80% acetonitrile in 0.1% trifluoroacetic acid. Peak fractions were concentrated on a SpeedVac 5301 (Eppendorf, Hamburg, Germany) for further analysis.

Immunoprecipitation

Four optic lobes were homogenized in 6 ml of 50 mM Tris buffer, pH 7.5 containing 5 mM EDTA, 2 mM DTT and protease inhibitors, 1 mM benzamidine, 2 µg/ml aprotinin and 0.3 mM PMSF, and centrifuged at 40,000 x g for 20 min. SDS to 0.3% was added to the supernatant and then heated to 60°C for 15 min. Two ml aliquots of the supernatant were incubated with 50 µL of Pansorbin cells (Calbiochem, Darmstadt, Germany) for 30 min at room temperature under gentle agitation. The material was centrifuged at 2,000 x g for 2 min and the pellet discarded. The sample was then incubated with 44 µg of C4 antibody or α-MVaMT for 2 h at room temperature and then re-incubated with 50 µL of Pansorbin cells for 30 min. After centrifugation for 2 min, the pellet was washed 3 times with Tris-buffered saline and extracted in 50 µL of SDS-PAGE sample buffer.

In situ trypsin digestion and mass spectrometry

The appropriate polypeptide band corresponding to p65 stained with colloidal Coomassie Blue (Imperial Protein Stain, Pierce) was excised from an 8% polyacrylamide gel (13 x 13 x 0.15 cm)

after SDS-PAGE. The protein band was washed 3 times with 50% acetonitrile in 0.1 M ammonium bicarbonate, dehydrated with acetonitrile and dried under vacuum. The protein band was rehydrated with 0.5 µg of trypsin in 20 µL of 0.1 M ammonium bicarbonate and submitted to *in situ* hydrolysis for 24 h at 37°C. Hydrolysis was stopped by addition of 5 µL of formic acid and the tryptic peptides were desalted in a microtip filled with POROS R2 resin (Perseptive Biosystem, Foster City, CA) previously activated in methanol and equilibrated with 0.2% formic acid, then washed 3 times with 150 µL of 0.2% formic acid. Peptides were eluted in 30 µL of 5% formic acid in 60% methanol. Mass spectrometry analysis of the tryptic peptides was carried out in an electrospray triple-quadrupole mass spectrometer Quattro II (Micromass, Manchester, UK) and MALDI-TOF-TOF Axima Performance (Kratos-Shimadzu Biotech, Manchester, UK). The sample was directly applied to electrospray ion source (ESI-3Q-MS) at 300 nL/min under the following conditions: capillary voltage maintained at 2.8 kV, cone voltage at 40 V and cone temperature set to 100 °C. In the mode of daughter ion scanning, the collision energy varied from 25 to 40 eV, and argon, at a partial pressure of 3.0×10^{-3} mTorr, was used as a collision gas. Each spectrum is an average of 20-50 scans (2 to 5 sec/scan) and processed using MassLynx software v.3.3. (Micromass). The mass/charge ratio of ions was deconvoluted to molecular mass using the MaxEnt3 algorithm. The amino acid sequences of tryptic peptides were deduced from series of *b* and *y* ions fragments produced by collision induced dissociation mass spectrometry (CID-MS/MS). The tryptic peptides of p65 were also analyzed by MALDI-TOF/TOF-MS using 4 hidroxy-cinamic acid as MALDI matrix (5 mg/ml) in 0.1 % TFA in 50% acetonitrile. Peptide sequencing was obtained by high energy collision induced dissociation at 20 keV and using helium as collision gas. All mass spectra from ESI-3Q and MALDI-TOF/TOF were submitted to databank search using MASCOT against nrNCBI, Swiss-Prot and squid ESTs.

Sequence analyses

The BLAST algorithms package (Altschul et al., 1990) was used locally in a Linux OS server to search for sequence similarities with the peptides extracted from the mass spectrometry analysis. BLASTp (peptides *versus* NCBI non-redundant protein database), tBLASTn (peptides *versus* *Loligo pealei* ESTs), and BLASTx (positive *Loligo pealei* ESTs matches *versus* NCBI nr protein database) searches were carried out using the most sensible parameters: *wordsize* = 2, low-complexity-regions filter off and *e-Value* = 10). Multiple sequence alignments were done using the ClustalW algorithm (Higgins and Sharp, 1989).

Cytoplasmic ribonucleoprotein preparation and RNase assay

Water for the solutions used here was treated with DEPC (1 ml per liter), left overnight and then autoclaved to destroy the DEPC. Ten optic lobes were homogenized in 3 ml of cold homogenization buffer containing 50 mM HEPES, pH 7.5, 125 mM NaCl, 100 mM sucrose, 2 mM potassium acetate, protease inhibitors and 40 units/ml of RNase Out (Invitrogen) with a motor-driven, Teflon-glass homogenizer by 10 strokes followed by a one min rest on ice and a second set of 10 strokes. The homogenate was centrifuged at 4,000 x g for 2 min and the supernatant (post-nuclear fraction) was centrifuged at 14,000 x g for 10 min. Three ml of this supernatant (post-mitochondrial fraction) was diluted with 4.5 ml of lysis buffer (83 mM Tris-HCl, pH 7.5, containing 1.7% NP40, 6.6 mM MgCl₂, 1.7 mM DTT and 75 µg/ml cyclohexamide). After 10 min on ice, 3 ml was applied to each of two discontinuous gradients made of 4.5 ml of 12 % and 34% sucrose in gradient buffer (50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 4 mM MgCl₂ and 1 mM DTT). The gradient was centrifuged at 202,000 x g in a swinging bucket rotor (40Ti, Beckman) for 90 min at 4°C. The fraction at the 12% sucrose upper interfase containing non-translated hnRNPs (Angenstein et al., 2005) was collected and diluted 1:1 in gradient buffer. Aliquots containing or not RNase (100µg/ml) were incubated for 30 min at room temperature and centrifuged at 400,000 x g (90Ti rotor, Beckman) for 20 min. Supernatant and pellet fractions were analyzed for the presence of p65 by western blots.

Immunohistochemistry

Fixed, 1mm transversal slices of optic lobes were included in Paraplast (Oxford Labwase, St Louis, MO) following the manufacture's instructions. Microtome slices of 10 μ m were cut, transferred to glass slides, de-parafinized and rehydrated by standard procedures. Slices were incubated in phosphate buffered saline (PBS) pH 7.4 containing 0.1 M glycine for 30 min at 4°C to block aldehyde groups and then washed 3 times for 10 min in PBS. They were then incubated in the dark at room temperature for 30 min in methanol containing 0.9% hydrogen peroxide solution to inhibit endogenous peroxidase activity, followed by washing in PBS. The samples were permeabilized and blocked by incubation in PBS containing 1% Triton X-100, 3% BSA and 0.5 % sheep serum and then incubated for 1 hr with primary antibodies (C4 diluted 1:10 and anti-synaptotagmin diluted 1:100 in PBS containing 0.1% Triton X-100, 3% BSA and 0.5 % sheep serum). After washing in PBS containing 0.1% Triton X-100, the slices were incubated for 1 hr with secondary antibodies conjugated to horse radish peroxidase (KPL, Gaithersburg, MD) diluted 1:400 in PBS containing 0.1% Triton X-100, 3% BSA and 0.5 % sheep serum and developed using 3,3'-diaminobenzidina (DAB) as substrate.

Immunofluorescence

Fixed stellate ganglia were directionally placed on and submerged in Tissue Tek (EMS, Hatfield PA). The blocks were frozen on acetone in dry ice and stored at -20°C. 10 micron slices were cut through the pre- and postsynaptic region of the giant synapse and placed on microscope slides. Fixed synaptosomes were adhered to glass microscope slides by incubation for 1 hr and gently washed with PBS. Slides containing either synaptosomes or stellate ganglia were then incubated in 0.1 M glycine, washed in PBS and blocked in 1mg/ml BSA, 1% goat serum and 1% Triton X-100 in PBS for 1 hr at room temperature. The slides were then washed with PBS containing 0.3% Triton X-100 and incubated with primary antibody in PBS containing 1mg/ml BSA, 1% goat serum, and 0.3% Triton X-100 for 2 hrs at room temperature. The slides were washed in PBS and incubated with appropriate secondary antibody conjugated to Alexa 488 for 1 hr

and washed again. In the case where double-labeling for squid synaptotagmin was done, the slide was incubated at this time for 1 hr with anti-syt-C2A conjugated to rhodamine and subsequently washed. The slides were mounted in Fluoromount G (EMS) diluted 2:1 in PBS and examined by confocal microscopy on a Leica Microsystems SP2 microscope (Mannheim, Germany).

Other methods

SDS-PAGE was performed using 4-20% linear gradient minigels, except where indicated. Western blots were on reinforced nitrocellulose membranes as previously described (Costa et al., 1999). Two-dimensional gels were done using the Ettan IPGPhorII Isoelectric Focusing System for the first dimension following the manufacturer's instructions (GE Healthcare) and SDS-PAGE, 8% large gels, for the second dimension.

Results

The C4 antibody is immunoreactive to p65 on immunoblots and by immunoprecipitation from extracts of squid optic lobes

Two affinity-purified, polyclonal antibodies raised against chicken myosin Va, one generated against the head domain (referred to as C4) and the other against the medial tail domain (α -MVaMT), both expressed in bacteria, were used in a western blot screen for interspecies immunoreactivity (Fig. 1A). A polypeptide corresponding to the myosin Va heavy chain (~200 k) was detected by α -MVaMT in extracts from mouse, rat and chick brain as well as from squid optic lobes. Although the C4 antibody was also immunoreactive towards myosin Va heavy chain in the vertebrates, it did not recognize the corresponding band in squid. However, it did strongly label a 65 kDa polypeptide (p65) in optic lobe extracts. This peptide was also immunoprecipitated from optic lobe extracts by the C4 antibody (Fig. 1B) but not by α -MVaMT. Squid myosin V was not immunoprecipitated by either of the antibodies. These results were the same if done on frozen optic lobes from *Loligo pealei* obtained from the Marine Biological Laboratory in Woods Hole, MA,

USA or from *Loligo plei* obtained from the Marine Biology Center of the University of São Paulo-CEBIMar, São Sebastião, Brazil and indicate that both denatured and cellular forms of p65 are immunoreactive to the C4 antibody.

p65 was purified by cation exchange and reverse phase chromatography

In order to identify p65, a purification protocol that started from an extract of optic lobes in 8 M urea was developed, which included ion-exchange chromatography on SP-Sepharose (supplementary Fig.S1) and reverse phase FPLC on a Source 5RPC column (supplementary Fig.S2). When the urea extract was applied to the SP-Sepharose column, p65 was not detected in the flow-through fraction and was eluted from the column in about 800 mM salt, indicating that it is a strongly basic protein. Since only a tenuous Coomassie-stained band could be detected in the peak fractions from the ion exchange column after SDS-PAGE that correlated to immunoreactivity on western blots (Fig. 2A), we conclude that p65 is not an abundant protein in optic lobes. In a fraction from the FPLC reverse phase column, however, p65 was isolated and concentrated, allowing it to be seen as a prominent protein band that was immunoreactive to C4 (Fig. 2B). This band was cut from the gel and analyzed by mass spectroscopy. Furthermore, on two dimensional gels (Fig. 2C and Supplementary Fig. S3) p65 was detected as a tight group of spots with pIs of 9.1 to 9.5, confirming its basic nature and revealing the presence of potential isoforms.

Peptide analysis indicated that p65 is a member of the hnRNP A/B subgroup of RNA binding proteins

Peptide sequences were obtained from mass spectroscopy analysis of the tryptic digestion of p65. Adjusting parameters for short input sequences we ran the BLASTp program against the NCBI non-redundant (nr) protein database (Table). This analysis suggested that p65 is an RNA-binding protein containing the well-defined, RNA recognition motifs, RNP-1 (RGFGFITY) and RNP-2 (LFIGGL), as well as glycine-rich sequences, commonly found in the C-terminal region of these

proteins. Sequences indicating a synaptic, calcium-binding motif and secreted protein with zinc finger motif were also found, suggesting a synaptic/exocytotic regulated function for p65. Since there are very few sequences corresponding to squid proteins in public data bases, we took advantage of a squid data bank in formation (DeGiorgis et al., manuscript in preparation) containing ~ 23,000 ESTs from *Loligo pealei* to search for nucleotide sequences corresponding to the p65 peptides. Running locally the tBLASTn algorithm we identified five ESTs that showed significant, but not exact, alignment with peptides 1 and 6 (Fig. 3). Using these five *Loligo pealei* ESTs in a BLASTx search against the NCBI nr protein database, we obtained strong correspondence to many hnRNPs, subtypes A/B, in species ranging from *Drosophila* to humans. This subset of RNA-binding proteins is also characterized by glycine-rich sequences in their c-terminal region, thus reinforcing the significance of peptides 4, 5 and 7 found in p65 (Table). Also, it has been reported that 21% of hnRNPs contain a zinc-finger domain (Maris et al., 2005), giving further significance to peptide 3 in the Table.

p65 is associated with hnRNP particles from cytoplasmic extracts in an RNA dependent manner.

In order to verify if p65 from tissue extracts is a cytoplasmic protein physically associated with RNA, a post nuclear and post mitochondrial fraction from optic lobes was obtained and applied on a sucrose gradient (Angenstein et al., 2005). The first interfase over 12% sucrose, enriched in hnRNP complexes, but not polysomes, contained p65 (Fig. 4, lane I). Since hnRNP complexes are labile to RNase treatment, this interfase fraction was collected, treated with RNase or not, and then centrifuged at high speed to separate the soluble and particulate fractions (Fig. 4). Without RNase treatment the pellet contained p65 whereas after treatment with RNase only traces of p65 were detected in the precipitated fraction, giving supporting evidence that tissue p65 is indeed part of a cytoplasmic hnRNP complex.

Immunohistochemistry and immunofluorescence studies indicated that p65 is found in presynaptic structures

To determine the localization of p65 in optic lobes, histological slices were incubated with the C4 antibody or anti-synaptotagmin and developed by the peroxidase-DAB method. Both antibodies labeled correspondingly-aligned bands within the outer plexiform layer (Fig. 5), which is a region of synaptic connections (Haghighat et al., 1984) particularly involving the photoreceptor terminations. Since synaptotagmin is a well-characterized, synaptic vesicle protein, the corresponding banding of the C4 antibody suggests a presynaptic location for p65 also. To further pinpoint the subcellular localization of p65, synaptosomes isolated from optic lobes were probed with C4 and control antibodies by western blots and by immunofluorescence microscopy. C4 clearly labeled p65 in blots of synaptosome extracts (Fig 6D). By immunofluorescence, large synaptosomes, 5-8 microns in diameter, showed intense granular staining by C4 frequently close to the plasma membrane, which correlated to subcellular structures imaged by bright field microscopy (Fig. 6A). Shown in Fig 6B and C are two controls – C5, an independent polyclonal antibody from a rabbit serum that was not immunoreactive to p65 on western blots (Fig. 6D) and the secondary antibody alone – that do not label the synaptosomes. Tissue slices through the giant synapse of the squid stellate ganglion showed intense labeling by anti-synaptotagmin, a marker for synaptic vesicles (Mikoshiha et al., 1995; Fukuda et al., 1995; Sugimori et al., 1998), on a strip between pre and post synaptic terminals (Fig. 7A), identifying it as the inner side of the presynaptic membrane and showing that it was accessible to the primary antibodies. At higher magnification with double labeling, irregular “puffy” dots were seen by anti-synaptotagmin (Fig 7B) suggestive of synaptic vesicle clusters, as expected for this protein. Strong staining for C4 was also observed over the pre-synaptic region (Fig 7C) although in smaller granules that frequently overlapped with the synaptotagmin clusters or were close near-neighboring particles to the clusters (Fig. 7D and E). The data confirm the presence of p65 at the presynaptic terminal and indicate a close spatial relationship to synaptic vesicle clusters.

Discussion

We have presented evidence that an affinity purified, polyclonal antibody, referred to as C4, raised against the head domain of chicken MVa is immunoreactive towards a highly basic, 65 k protein (p65) purified from squid optic lobes. Since this antibody had a high degree of specificity for p65 in squid extracts but was not reactive towards squid myosin V, we sought to identify p65 by biochemical and immunological techniques. Peptide sequences, obtained from purified p65 by mass spectrometry and analyzed by BLAST algorithms, indicated that p65 is an RNA-binding protein, member of the hnRNP family, subtype A/B. Characteristics of this subtype are a basic protein having pIs of 8-9, the presence of two or more RNA recognition motifs (RNP-1 e RNP-2) and a glycine-rich accessory domain. About 21% have additional motifs such as zinc finger domains (Maris et al., 2005). All of these features were found in p65. Immunolocalization studies showed p65 within the outer plexiform layer and in large presynaptic terminals biochemically isolated from the photoreceptor neurons of squid optic lobes, as well as being closely associated with synaptic vesicle clusters at the presynaptic terminal of the giant synapse in the stellate ganglion. Thus, we have identified a novel 65 k RNA-binding protein, present in squid presynaptic terminals.

A first question is why would an affinity-purified antibody raised against chicken myosin Va specifically recognize a non-related protein in squid neuronal tissue. Irrelevant, random, cross reactivity may be the simple answer and further speculation, at first thought, not worth pursuing. However, curiously, a polyclonal antibody, raised against the peptide LASNPIMESFGNAK found in the head domain of squid myosin V, weakly labeled purified squid myosin V on western blots and strongly labeled a “55 k protein” on these same blots (Molyneaux and Langford, 1997), apparently a contaminant in the myosin V preparation, as commented by these authors. This peptide from squid is highly conserved and is identical, except for a single amino acid substitution (F to I at peptide position 10), to one found in the head domain of chicken MVa. Since the C4 antibody used in the present work was raised against the head domain of chicken MVa, the same, or practically the same, antigenic determinant was present for the generation of both the squid peptide antibody and C4. We speculate that the “55 k immunoreactive protein” seen by Molyneaux and Langford is the

same as the p65 identified here, which leads us to suggest that there is, in fact, a defined structural basis for this cross reactivity. With the present information in the public data banks a BLAST search with this peptide gave only myosin V hits, nothing that hinted of RNA-binding proteins. Full sequence determination of p65, now in progress, may give clues as to the nature of the antigenic determinant and possible evolutionary significance.

The suspicion of a structural basis for the cross reactivity described here and of possible evolutionary relevance is augmented by recent evidence that myosin V is involved in the transport and cytoplasmic localization of mRNAs; thus far, the only myosin class shown to have this function. The first and most detailed example in the literature is the role that yeast Myo4p, one of two myosin Vs in *Saccharomyces cerevisiae*, plays in the translocation of ASH1 mRNA into the bud tip (Gonsalvez et al., 2005; Pruyne et al., 2004; Muller et al., 2007). Recently, the other yeast myosin V, Myo2p, has also been implicated as a molecular motor component of a large messenger ribonucleoprotein (mRNP) complex associated with cytoplasmic P-bodies (Chang et al., 2008). In vertebrates, MVa has been associated with several RNA binding particles, for example, a mRNP complex containing Pura, mStaufen and Fragile X Mental Retardation Protein (Ohashi et al., 2002). Also, periaxoplasmic ribosomal plaques, discrete ribosome-containing domains found along myelinated axons, contain both MVa protein and its mRNA (Calliari et al., 2002; Sotelo-Silveira et al., 2004). Finally, MVa has been shown to associate with TLS (*translocated in liposarcoma*), a RNA-binding protein that is part of a large mRNP complex involved in transport and translation of mRNAs into dendrites (Yoshimura et al., 2006). In MVa-deficient hippocampal neurons, TLS cannot accumulate in spines upon glutamate receptor activation. Thus, these data indicate that there are functional associations between MVa, RNA, and RNA-binding proteins.

The hnRNPs form a large, diverse and structurally conserved family of proteins that are essential components of the multi-component RNA-protein complexes involved in the processing, regulation, translocation and stability of mRNAs (Maris et al., 2005; Dreyfuss et al., 2002; Dreyfuss et al., 1993). Members of the A/B subset of hnRNPs are highly conserved from invertebrates to mammals and characteristically shuttle between the nucleus and cytoplasm, but also are involved in the localization

and stability of mRNA and regulation of its translation at distant subcellular sites. In neurons, many different mRNAs encoding synaptic proteins are targeted to the synaptic region and translated locally. Upon arrival, at least two events must occur: the liberation of specific mRNAs from granule repression and the activation of the local synthesis machinery. For example, in mammals hnRNP A2 is an essential component in a large multi-component granule that transports mRNAs away from the nucleus along microtubules out to dendrites (Carson et al., 2008). Translation of these mRNAs during transport is repressed by another RNA-binding protein, hnRNP E1. Once at their destinations, local control over translation of these transported mRNAs has been related to synaptic activity and plasticity. The mRNA encoding calmodulin-dependent protein kinase II (CaMKII), for example, is transported via this A2 pathway to dendrites where its local synthesis, stimulated by NMDA receptor activation, is required for long-term potentiation (Miller et al., 2002). Antagonists of the NMDA receptor inhibit CaMKII synthesis (Wells et al., 2000), thus illustrating the link between synaptic activity, local protein synthesis and synaptic plasticity. It is remarkable that an increase in the synthesis of CaMKII upon tetanic stimulation has been detected as early as 5 min post-stimulation in hippocampal slices (Ouyang et al., 1999). Similarly, a type of long-term depression, triggered by metabotropic glutamate receptor activation in hippocampal cells, requires the rapid translation (within 60 min of activation) of preexisting mRNA in the postsynaptic dendrites (Huber et al., 2000).

These examples of activity-related control on synaptic protein synthesis have mostly been demonstrated in dendrites (Wells, 2006). There is still a paucity of information on the function and regulation of the translation of the numerous mRNAs that have been identified specifically in the presynaptic terminals. (Gioio et al., 2004; Kaplan et al., 2004; Giuditta et al., 2002; Jimenez et al., 2002; Sotelo-Silveira et al., 2006; Alvarez et al., 2000), although invertebrate models offer some information. Long-term sensitization in *Aplysia* neurons elicited by serotonin was blocked by protein synthesis inhibitors injected into the sensory neuron but not when injected into the postsynaptic motor neuron (Martin et al., 1997). Furthermore, levels of protein synthesis in synaptosomes from squid optic lobes were altered by manipulation of intracellular calcium levels (Benech et al., 1999). These examples support the hypothesis that local translation and regulation of specific mRNAs at the presynaptic

terminal is intimately related to synaptic activity and plasticity, as has been more clearly evidenced in dendrites. Based on this panorama and on our data presented here, we suggest that p65 is a novel link at the presynaptic terminal between local RNA processing and synaptic function, and as such represents an important new lead towards factors involved in synaptic plasticity.

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Table. Identification by mass spectrometry of tryptic peptides from p65. Purified p65 was excised from an 8% SDS-PAGE gel, digested with trypsin and analyzed by mass spectroscopy as described in Materials and Methods. Periods before or after the sequences in the table indicate that the extremities of the peptide could not be determined from the spectra.

peptide	peptide mass Mr	sequence	BLAST search description
1	931.43	GFGFITYK	hnRNP; glycine-rich RNA binding protein
2	1105.0	LSTLDPESDK	Ca ²⁺ -dependent lipid binding protein; Ca ²⁺ -binding motif in phospholipase, prot.kinase C and synaptotagmin
3	1459.65	YPSYGDSYGGGLPK	Zinc finger; secreted protein with zinc-dependent carboxypeptidase domain
4	1673.61	.GGGGGGGGGGGG.	Glycine-rich RNA-binding protein; RNP complex protein
5	1730.64	.GGGGGGGGGGGG.	Glycine-rich RNA-binding protein; RNP complex protein
6	1913.92	LFIGGLSYDTNEDTIKK	Cold-inducible RNA binding protein
7	2914.29	.PWSFPGGGGYGGGGNFQ.	Cellular nucleic acid binding protein; glycine-rich RNA binding protein; cytoplasmic poly-A binding protein 4

Figure legends

Fig. 1. The C4 polyclonal antibody recognizes a 65 k polypeptide in squid optic lobe extracts.

(A) Western blots of tissue extracts from mouse, rat and chick brains, and squid optic lobes, probed with a polyclonal antibody raised against the head domain of chicken MVa (C4, upper panel), or a polyclonal antibody raised against the medial tail domain, anti-MVa-MT (α -MT, lower panel), respectively. To the right of the blots, the M_r positions of the heavy chain of myosin Va (hcMVa) and p65 are indicated. (B) Immunoprecipitation of p65 from squid optic lobe extracts by C4. Extracts of squid optic lobes were subjected to immunoprecipitation as described in Experimental Procedures and analyzed by Western blots probed with the C4 antibody. S1, input fraction; lane 1, fraction immunoprecipitated by C4 antibody; lane 2, fraction immunoprecipitated by α -MVa-MT; lane 3, fraction precipitated from the extract without antibody. The positions of p65 and IgG heavy chain are indicated to the right of the figure.

Fig. 2. p65 is a low-abundant, basic protein comprised of isoforms. (A) SDS-PAGE of the peak

p65 fraction (38) from the SP-Sepharose column (see Supplementary Fig.S1) stained with Coomassie Blue-R (CB) and western blot (C4) probed with the C4 antibody. Molecular mass markers are indicated to the left. (B) SDS-PAGE on an 8% gel of fractions 13 and 14 from the reverse phase chromatography (5RPC) (see Supplementary Fig.S2) stained with colloidal Coomassie Blue (CCB) and western blot (C4) probed with the C4 antibody. Molecular mass markers are shown and indicated to the left. (C) A section of a 2-D gel stained with silver of fraction 13 from the reverse phase column (left panel) and western blot of an equivalent gel probed with the C4 antibody (right panel). The pI range is given above the sections and potential isoforms are numbered 1-5. (The complete 2-D gel is shown in Supplementary Fig. S3)

Fig. 3. Identification of ESTs in the *Loligo pealei* data base with peptides derived from p65.

The open reading frames of ESTs from the *Loligo* data bank were converted to amino acid sequences using the tblastn program available from the NCBI site. Alignment between multiple sequences was performed via the ClustalW program. Only sequences 1 and 6 from the Table had

significant homology within the available ESTs and their alignment with 5 ESTs are shown in the figure. The boxed sequences illustrate the consensus sequences for RNP 1 and RNP 2, as indicated. Identity between the peptide amino acids and at least 4 out of the 5 EST sequences is indicated by an asterisk below the alignment and chemically homologous amino acids by two dots.

Fig. 4. Biochemical evidence that p65 is associated with cytoplasmic RNP particles. SDS-PAGE gel stained with Coomassie blue-R (upper panel) and western blot probed with C4 (lower panel) of a fraction (I) from optic lobe extracts prepared to preserve mRNP particles as described in Experimental Procedures. Aliquots of this fraction were treated with RNase (+) or water (-), incubated for 30 min and then centrifuged at 400,000 x g for 20 min. (S) and (P) indicate the supernatant and pellet fractions, respectively. p65 is indicated to the right of the bottom panel.

Fig. 5. Immunohistochemistry of slices from squid optic lobes. Transversal, 10 µm slices through the squid optic lobe were labeled with C4 (A), anti-synaptotagmin (B) or secondary antibody alone (C) by the peroxidase-DAB method as described in Experimental Procedures. The arrows indicate the immunopositive bands for both primary antibodies in the outer plexiform layer (opx). The morphological layers of the optic lobe cortex as described by Haghighat et al. (1984) are indicated to the right: outer nuclear (on), outer plexiform (opx), inner nuclear (in), inner plexiform (ipx) and mononuclear (mn) layers.

Fig. 6. Immunofluorescence labeling of synaptosomes from squid optic lobes. (A) A gallery of confocal images of synaptosomes immunolabeled with C4 primary and Alexa 488 secondary antibodies (green) next to their DIC images is illustrated. (B) Control showing synaptosomes probed with a rabbit polyclonal antibody, C5, that was not immunoreactive to p65 on western blots, or (C) probed with the secondary antibody conjugate, anti-rabbit IgG/Alexa 488, alone. The scale bars represent 5 microns. (D) Western blots of extracts from synaptosomes stained with Coomassie blue (CB) or probed with C4 or C5 antibodies, as indicated, or secondary alone (2nd). Molecular mass markers are indicated on the left.

Fig. 7. Immunofluorescence labeling of the giant synapse of the stellate ganglion. A transversal histological slice of 10 microns through the stellate ganglion showing pre- and postsynaptic terminals of the giant synapse was double-labeled with the antibody C4 (green) and anti-synaptotagmin (anti-syt-C2A, red). **Control** section was probed with only the Alexa 488 (green) conjugated secondary antibody, without primary antibody. **(A)** A low magnification image illustrating the localization of synaptotagmin on a strip between the pre- and postsynaptic terminals (pre and post, respectively). **(B, C and D)** Higher magnifications of the presynaptic strip with double labeling. Irregular clusters of presumed synaptic vesicles are labeled by anti-syt-C2A (red) whereas smaller particles distributed over the same area are labeled by C4 (green). **(E)** A blow up of the boxed area in D. The merged images (D and E) show frequent punctual overlap (yellow), some of which are indicated by arrows, as well as close contact (arrowheads) between these markers. The scale bars represent 10 microns.

Interspecies immunoreactivity of MVa antibodies

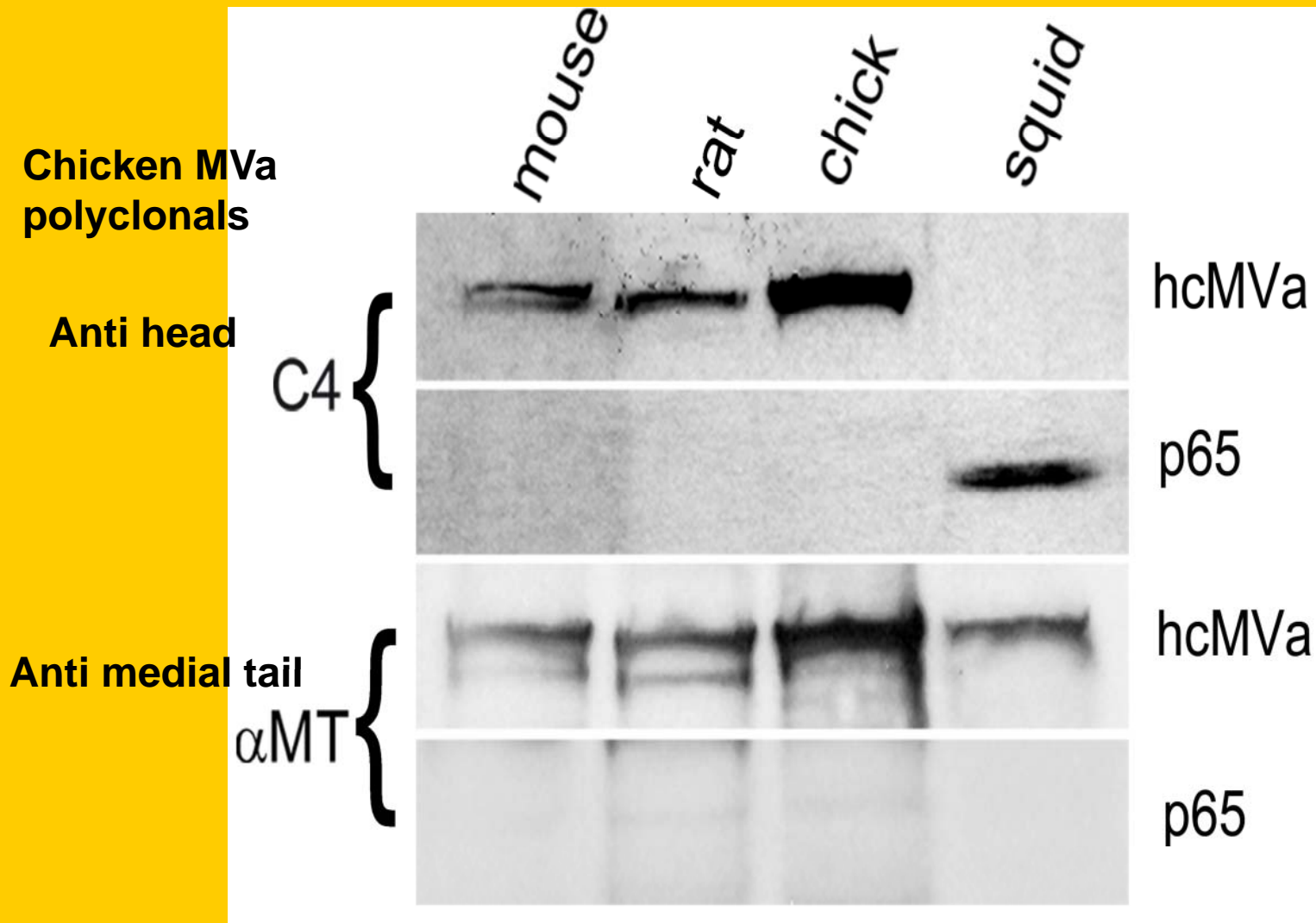


Figure 1A

Immunoprecipitation of p65 from squid optic lobes

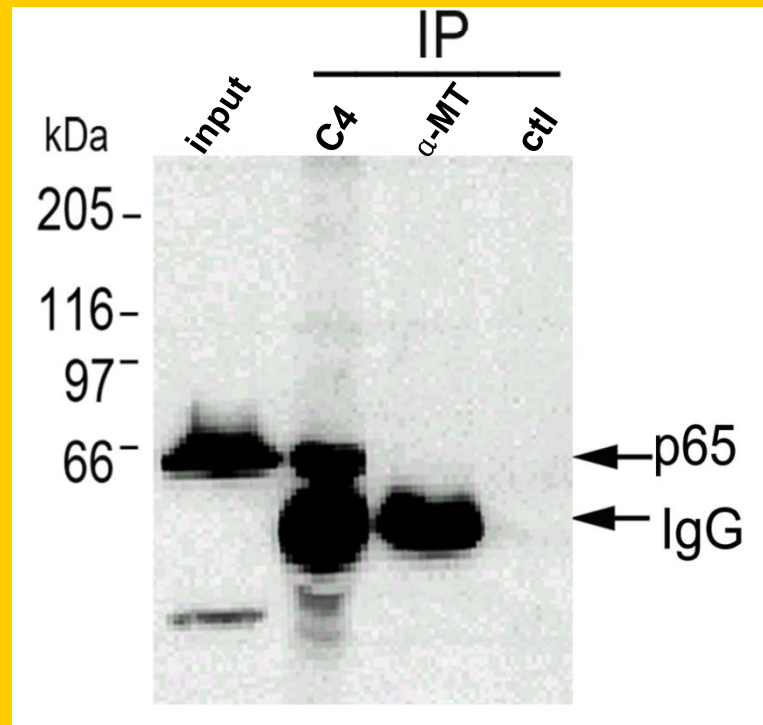
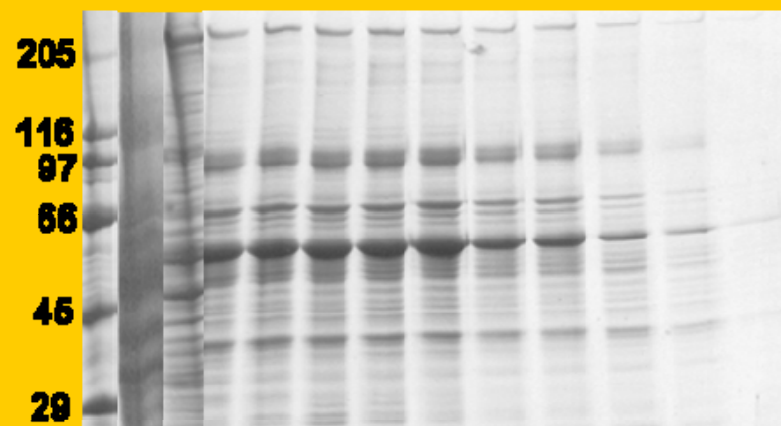


Figure 1B

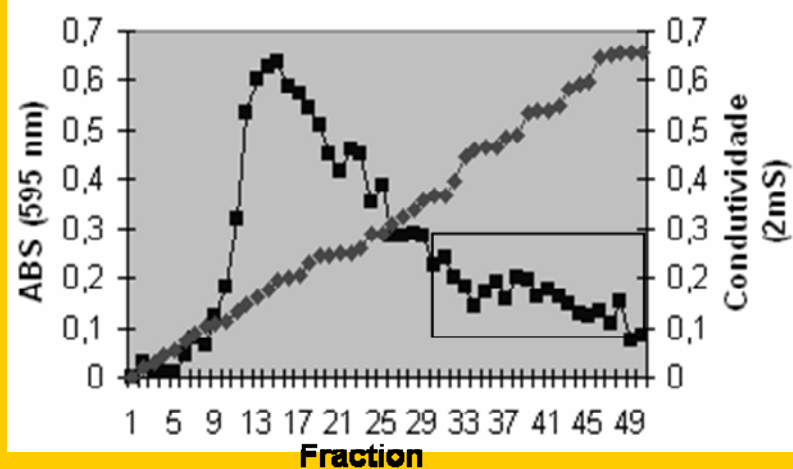
Cationic exchange

B kDa S2 FT 32 34 36 38 40 42 44 46 48 50

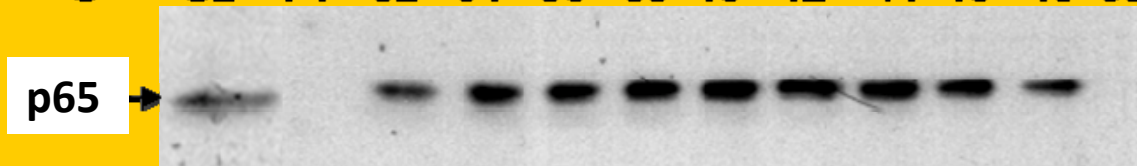


SDS-PAGE

SP-Sepharose



C S2 FT 32 34 36 38 40 42 44 46 48 50

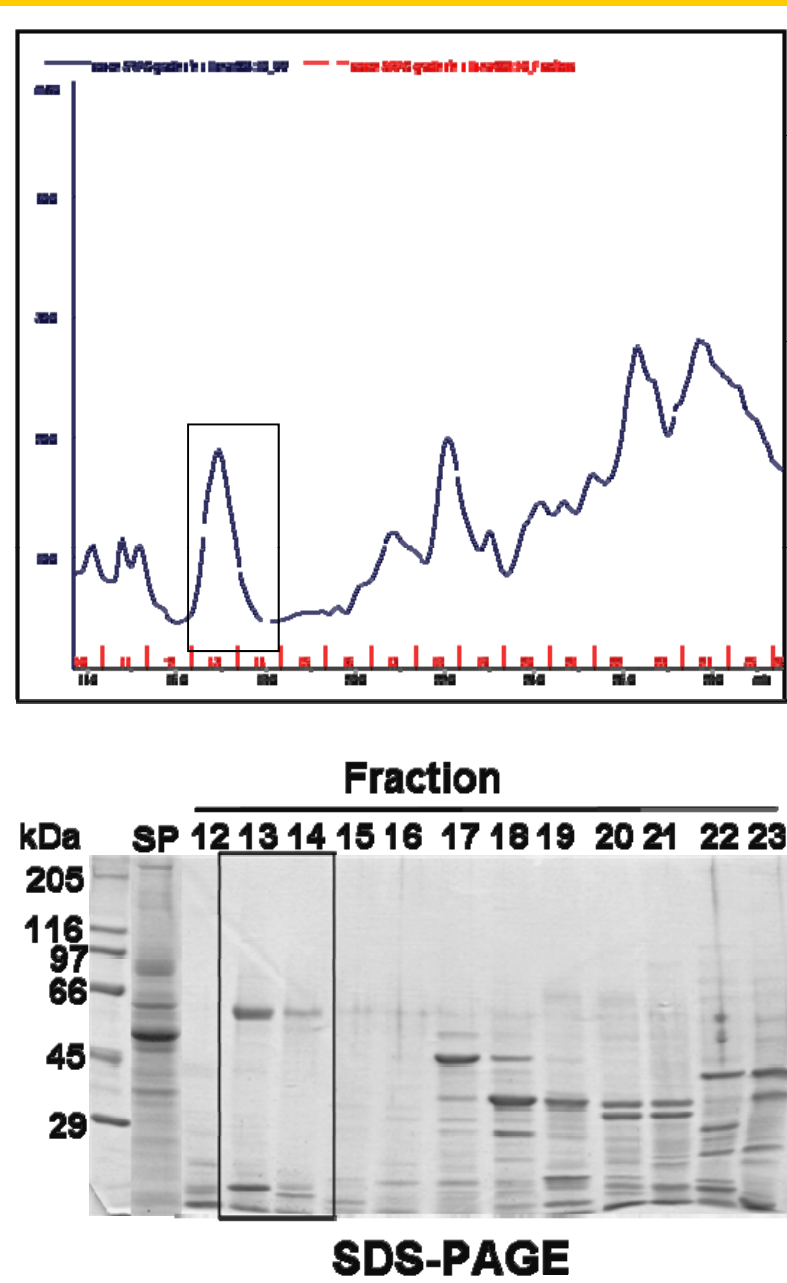


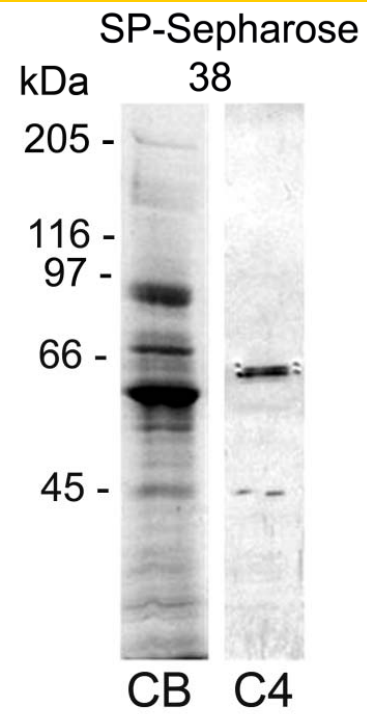
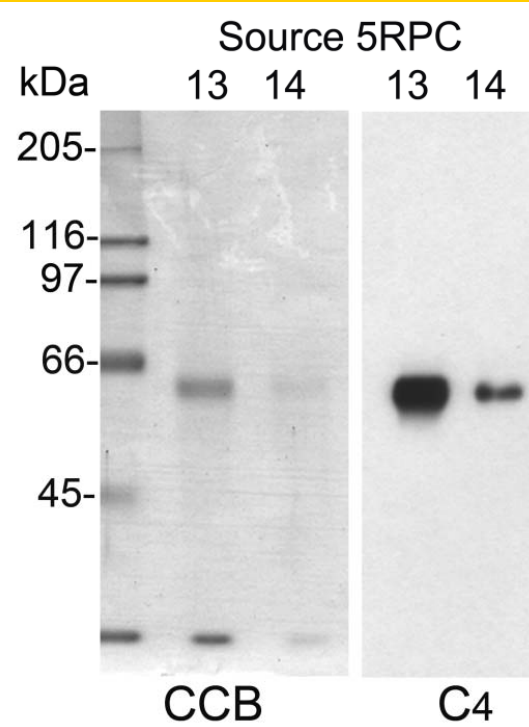
Western blot

Figure S1

FPLC reverse phase

Figure S2



A**B****Figure 2**

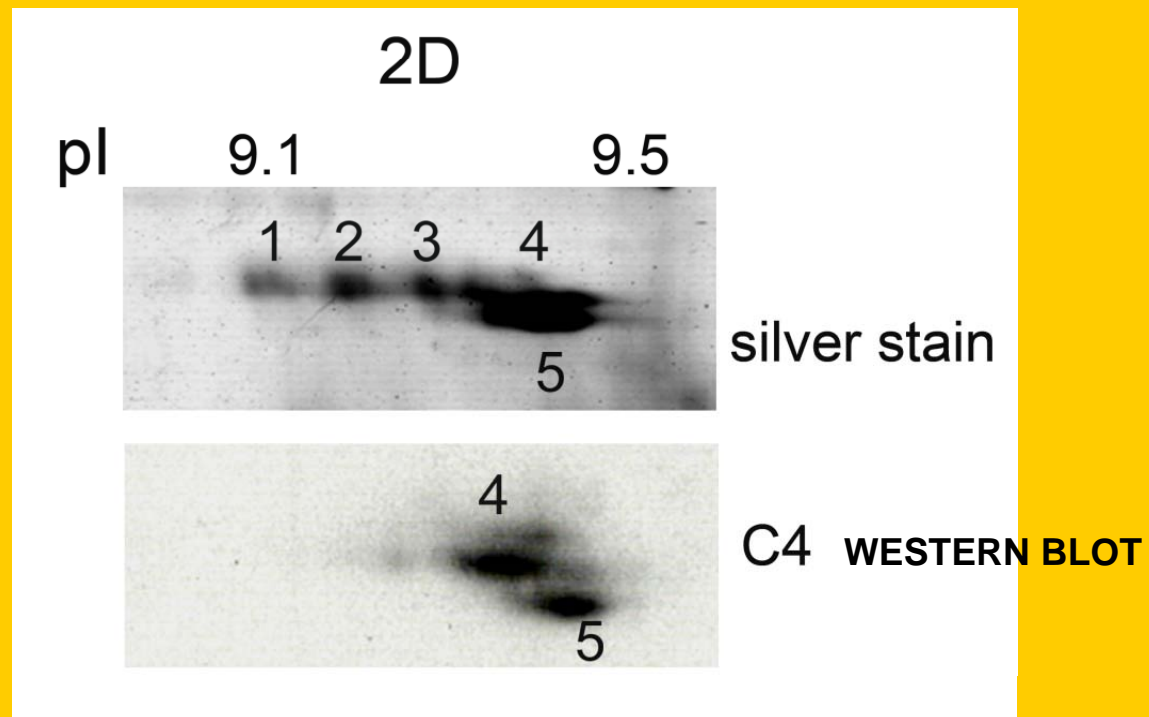


Figure 2C

ESTs from Loligo pealei

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peptide -----
oy189g03.y3 -----
oy195d11.y1 -----VRPRVRLTSVLGSLSLSTTFNPSSLSGDIFLPCRGGFFFPFILFSNNTGLADNFF
oy189c02.y3 -----PKGAIMP-----
oy252h05.y1 -----ETLTQFSSVSTRTESKRELSEGKREKNWRTYIYR-----
oy225b06.y1 GSEHIRHLCIFLLGFRLLYHKTEKTQKKLVSLIFPPLSSFTMTDFQTDPEVMDGENGAN

                                RNP 2
peptide -----KLFIGGLSYDTNEDTIKK-----
oy189g03.y3 -----DDNDPQAEKFRKLFIGGLNYDTTEETIKQHFEQWG
oy195d11.y1 LKRLTKTLFFGLQNMFKRMENGT SNHNDDPNAEKFRKLFIGGLNYDTTEDSLRITYFEQWG
oy189c02.y3 -----ESTVRYRSDSDGEKFC KLFIGGLNYTTNEEAMKEYFEPWG
oy252h05.y1 -----YRLTHLYSKMPEFDQEQRYRDGEEKYCELFIGGLNYTATQEGLRGRFEQWG
oy225b06.y1 NIDGHENSEEVVETFEAMETMDGDDKGNDDEESRKLFVGGLSWETKAKDLREYFFKFG
                                *****: * * : ::

                                RNP 1
peptide -----RGFGFITYK-----
oy189g03.y3 EIVDCVVMKNPATKKS RGFGFITYKAAEMLDDAQTNRPHKIDNRELDTKRAMPRNESDES
oy195d11.y1 EVVDCVVMKNPSTNRSRGFGFITYQSAEQLDDAQTNRPHIDSKKLDTKRAMPRDGSEET
oy189c02.y3 EVVDCVVMRDNTKKS RGFGFITYKTEEQVDEAQRNRPHNIDNKEVETKRAMPRNETD-S
oy252h05.y1 EIVD-DKMRDPVTG-GRGFGFITYKTEEVLDDAHKNRPHKLDGTEVDTQRAIPQNESDEA
oy225b06.y1 EVEHCNLKTDPVTKRSRGFGFVLFKEATVDKALLEKEHKLHGRNIDPKRANPR-----
                                *****:***

                                RNP 2
peptide -----KLFIGGLSYDTNEDTIKK-----
oy189g03.y3 QTSVKKMFVGGGLKDDTAEDDVREVFGRFGKIEKLEMIKDKNKGKRGFCFITFDDFDCVD
oy195d11.y1 QASVKKMFVGGLEETTSEDEIRDIFSKYGTVEKVDMIKDKNKGKQKRCFVTFDDFDAVD
oy189c02.y3 QATVKKLFVGGIKEDTSEEEIREFFSTKGKIESIDMITDKGTGKKRGFCFITFEDYDSTD
oy252h05.y1 QATVEKMFVGGGLTDGTTEEEIECKFETFGIIDKVEMIK-----
oy225b06.y1 -EPIKKVFVGNLDQNLTEIREYFGKFGKITKLELPFDKMRNQRRNYCFIEFES-----
                                *:***** * *: *:

peptide -----
oy189g03.y3 KCVLKRRIFPHGKYVEV-----
oy195d11.y1 KCVIRKRFPLGSKSI-----
oy189c02.y3 KLVLKKYLDPKGRVEVKLLSELK
oy252h05.y1 -----
oy225b06.y1 -----

```

hnRNP A/B

Figure 3

P65 IS ASSOCIATED WITH CYTOPLASMIC RNP PARTICLES VIA RNA

Post-nuclear, post-mitochondrial fraction
Applied to discontinuous sucrose gradient

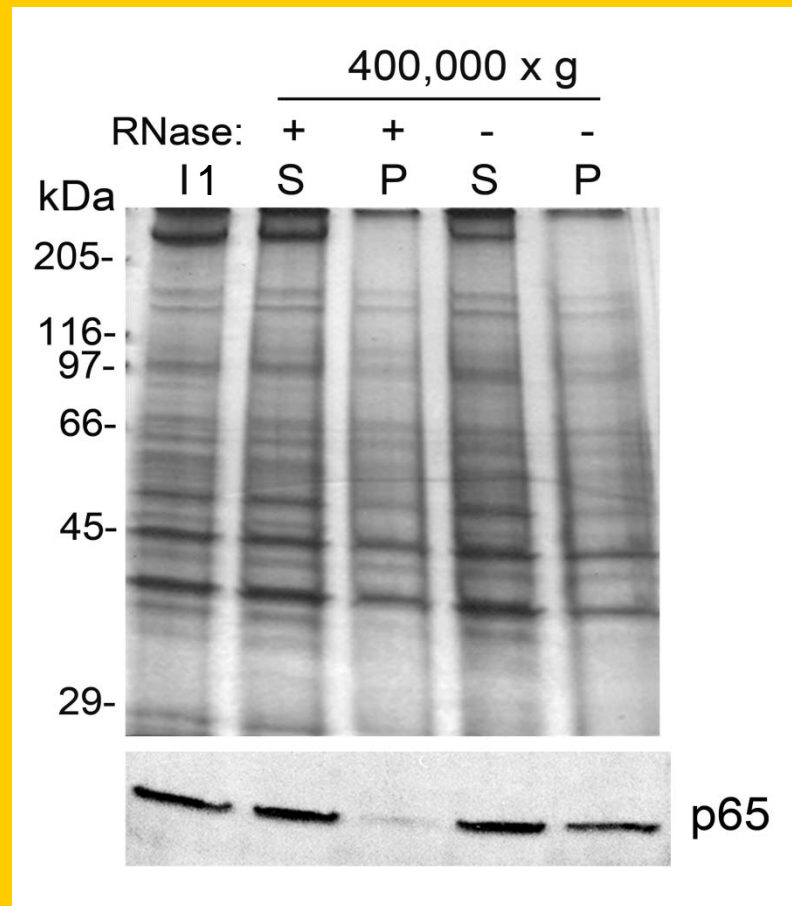
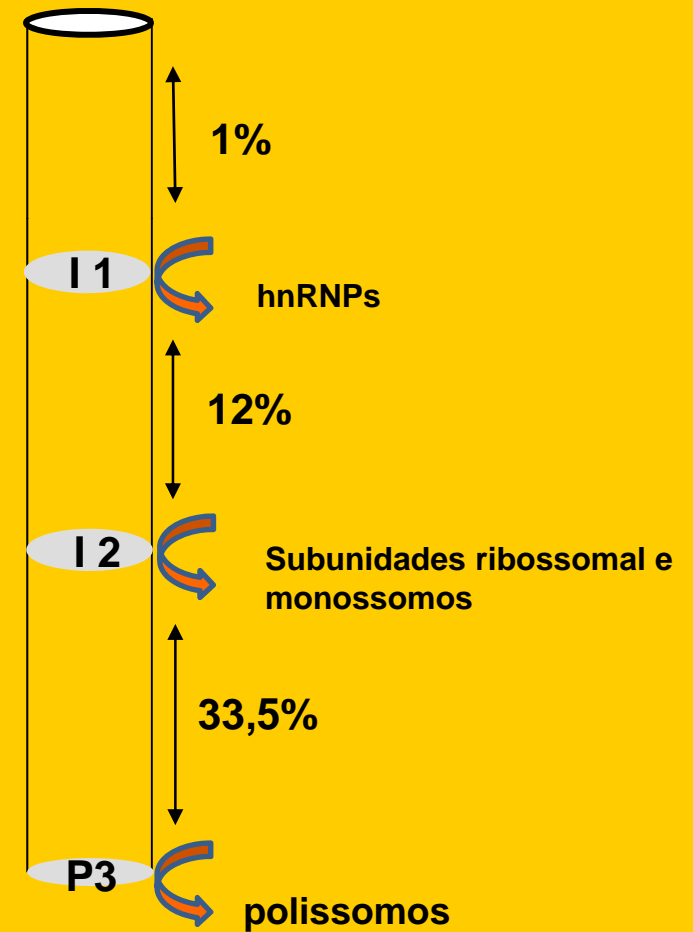


Figure 4



Immunohistochemistry of slices from squid optic lobes

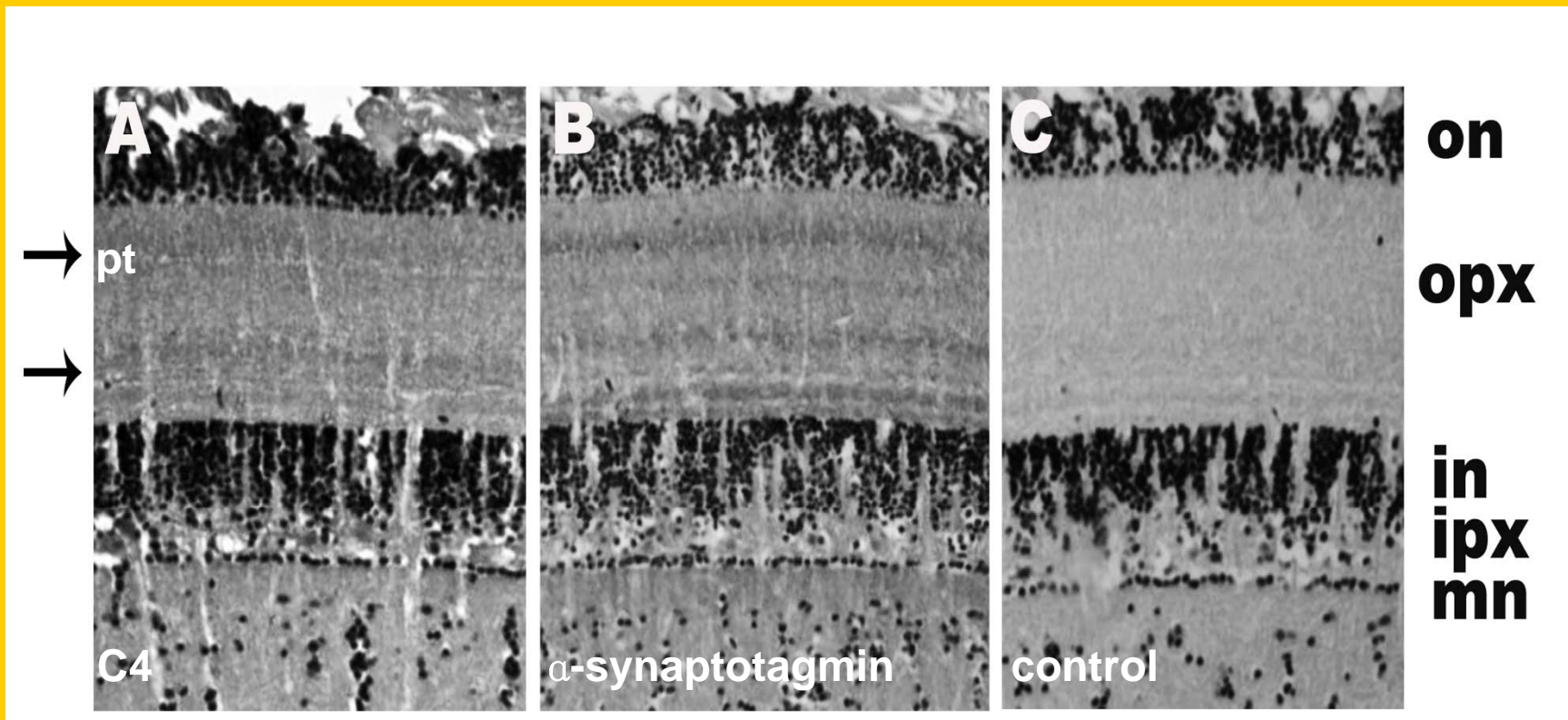


Figure 5

outer nuclear (on)
outer plexiform (opx)
inner nuclear (in)
inner plexiform (ipx)
mononuclear (mn) layers

C4 immunolabeling of squid optic lobe synaptosomes

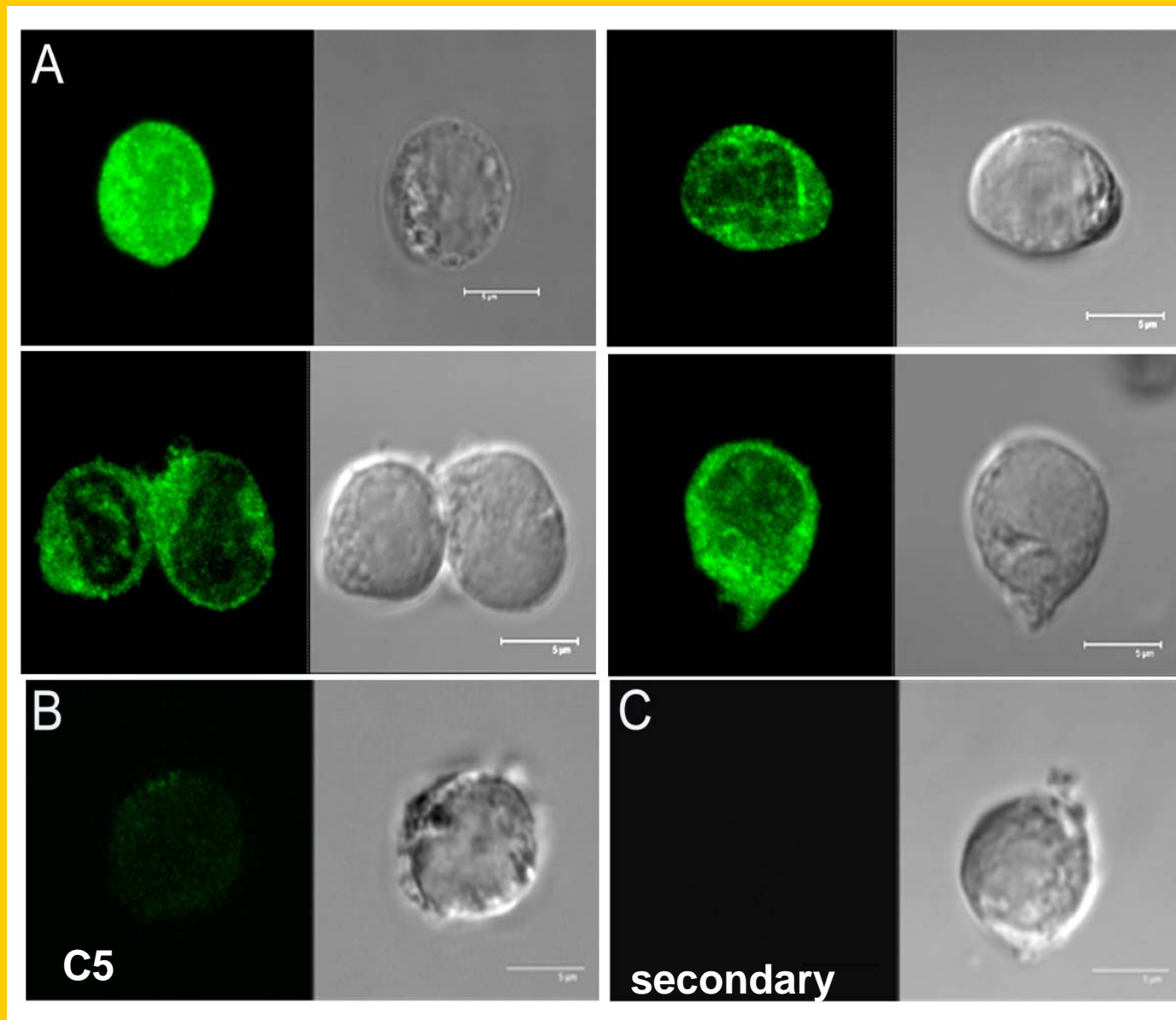


Figure 6

Synaptosomes from squid optic lobes

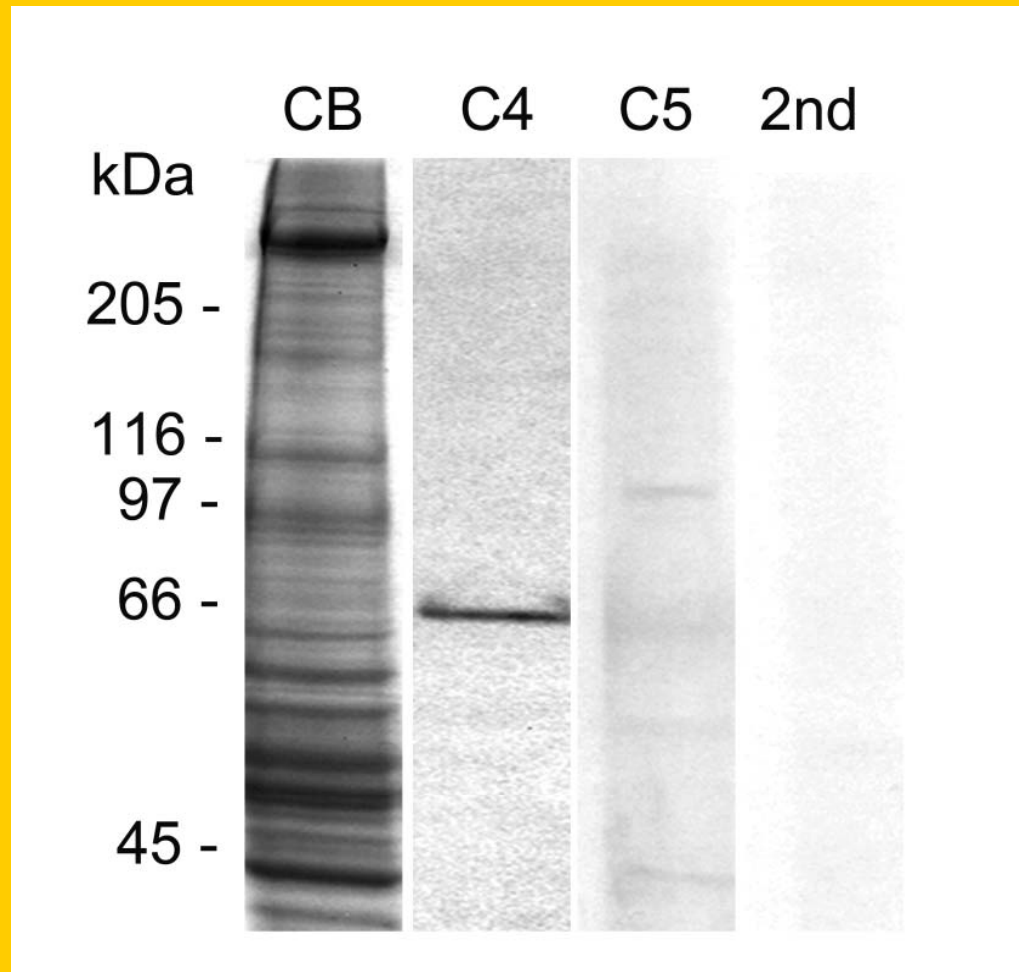


Figure 6D

Stellate ganglion Giant synapse

Synaptotagmin (red)
p65 (green)

Figure 7

